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In re Application of: Suheir ASSADY et al.

Confirmation No. 4072

Application No.: 10/714,348

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Examiner:

For: INSULIN PRODUCING CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

Atty. Docket No.: 85189-5400

SUBMISSION OF CERTIFIED PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

Applicants have claimed priority of Israeli application no. IL143155 filed May 15, 2001, under 35 U.S.C. § 119. In support of this claim, a certified copy of said application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

Date: 8/31/04

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Attorney Docket No.	Appln. Serial No./ Patent No.	Items - Documents filed on **August 31, 2004**	Patent Fees- Acct. #50-1814
85189-5400	**10/714,348**	**Submission of Certified Priority Document App. No. IL143155**	**0**

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חוק הפטנטים, תשכ"ז - 1967
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בקשה לפטנט
Application for Patent

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Insulin Producing Cells Derived from Human Embryonic Stem Cells

RAP/002

Insulin Producing Cells Derived From Human Embryonic Stem Cells

Field of the Invention

The present invention relates to insulin-producing human embryonic stem cells, to the generation and enrichment of populations of insulin-producing human embryonic stem cells, to isolation of insulin-producing human embryonic stem cells or stable cell lines and to methods of using these cells, particularly for cell replacement therapy.

Background of the invention

Type 1 diabetes mellitus generally results from autoimmune destruction of pancreatic islet β-cells, with consequent absolute insulin deficiency and complete dependence upon exogenous insulin treatment. The relative paucity of donations for pancreas or islet allograft transplantation has prompted the search for alternative sources for β-cell replacement therapy.

Recent studies have emphasized the importance of strict glycemic control in order to reduce ophthalmologic, neurologic, and renal complications of Type I diabetes mellitus (1). Yet, pancreatic and islet cell replacement is currently considered to be the only truly curative approach. Indeed, this approach was recently shown to reverse glomerular lesions in patients with diabetic nephropathy (2). The promise of this approach has recently been further strengthened by a report of the use of an improved, less hazardous glucocorticoid-free immunosuppresive regimen in islet allograft transplantation (3). However, the shortage in donations is a primary obstacle, preventing this approach from becoming a practical solution. Thus, attention has focused on the

use of alternative sources such as xenografts, which have other disadvantages, including the potential risk of undetermined zoonotic infections (4). It has also been suggested that β-cell lines derived from rodents might provide an unlimited source for cell replacement therapy. In addition to the problem inherent in xenobiotic sources, such cell lines have been shown to display phenotypic instability, with loss of insulin biosynthesis and regulated secretion while proliferating (5-7). Another more recently described approach involves extending the β-cell phenotype to other tissues using *in vivo* gene transfer (8,9), either by expressing the insulin gene or an insulin gene analogue under the control of a glucose sensitive promoter, or by ectopic expression of insulin promoter factor1/pancreatic and duodenal homeobox gene 1 (IPF1/PDX1) (10).

The establishment of pluripotent human embryonic stem (hES) cells (11,12) and embryonic germ (EG) cells (13), have introduced a new potential source for cell therapy in type 1 diabetic patients, especially in the light of recent successes in producing glucose-sensitive insulin-secreting cells from mouse ES (14). hES cells grow as homogeneous and undifferentiated colonies, when they are propagated on a feeder layer of mouse embryonic fibroblasts (MEFs) (11). As previously shown, they have a normal karyotype, express telomerase and embryonic cell surface markers. Removal from the MEF feeder layer is associated with differentiation into derivatives of the three embryonic germ layers, as evident from teratomas formed following subcutaneous injection in nude mice (11). Endodermal markers, but not insulin expression, were reported in a previous general survey of different growth conditions and differentiation markers in EG cells (15). Using RT-PCR applied to RNA

extracted from differentiated hES cells, detection of a variety of differentiated cell markers including insulin was reported (16).

Various methods for generating novel embryonic cell populations, for propagation of embryonic stem cells utilizing combinations of growth factors and for immortalization of such cell lines are known in the art, as for example disclosed in US 5,690,926, EP380646, and US 6,110,739, among many others. Nowhere in the background art is it taught that insulin-producing cell lines may be derived from established human stem cell lines.

Summary of the Invention

It is an object of the present invention to provide insulin-producing cells derived from human embryonic stem cells. It is yet another object of the present invention to provide cell populations enriched for insulin-producing cells derived from human embryonic stem cells. Preferably cell populations containing selected insulin-producing cells derived from human embryonic stem cells, more preferably isolated cells or most preferably cloned cell lines will be provided in accordance with the principles of the present invention.

According to another aspect of the present invention insulin-producing cells derived from hES cells or cell populations comprising insulin-producing cells derived from hES cells will be regulatable. Preferably they will be glucose responsive, or glucose regulatable in terms of the insulin production and secretion.

According to yet another aspect of the present invention the insulin-producing cells derived from hES cells or cell populations comprising insulin-producing cells derived from hES cells will be stable over prolonged periods of time,

preferably they will be long-lived, i.e., will not undergo growth arrest or senescence, more preferably they will be stable cell lines, most preferably they will be stable clonal cell lines, alternatively they will be immortalized cell lines. According to yet another aspect according to the present invention, the insulin producing cells derived from hES cells or cell populations comprising insulin-producing cells derived from hES cells will be useful for medical applications, including but not limited to cell replacement therapy.

According to the principles of the invention it is now disclosed that pluripotent undifferentiated human embryonic stem (hES) cells can serve as a system for lineage specific differentiation. In a currently preferred embodiment using hES cells in both adherent and suspension culture conditions, it was demonstrated that *in vitro* differentiation included the generation of cells with characteristics of insulin producing β-cells. Immunohistochemical staining for insulin was observed in a surprisingly high percentage of cells. Secretion of insulin into the medium, was observed in a differentiation-dependent manner, and was associated with the appearance of other β-cells markers. These findings validate the hES cell system, as a potential basis for derivation and/or enrichment of human β-cells or their precursors as a possible source for cell replacement therapy in diabetes.

Brief description of the Drawings

Figure 1

Differentiation of human embryonic stem cells in suspension culture.

a) Simple embryoid body, three days after removal of MEF and growing in suspension culture. Embryoid bodies (EBs) were collected every three days, fixed in 10% neutral buffered formalin, dehydrated in graduated alcohol and embedded in paraffin. 5□m sections were stained with hematoxylin and eosin; **b)** Day 3; **c)** Day 17 after differentiation. Original magnification, (a) 20x; (b,c) 40x.

Figure 2

Insulin-expressing cells in embryoid bodies. Immunohistochemistry was performed on paraffin sections as described in methods. **a)** Normal human pancreas was used as a positive control, 40x magnification; **b-d)** EBs at day 19 after differentiation, original magnification 40x; **e)** EB at 19 days after differentiation at 100x magnification, in which the plane of section shows cells in which the cytoplasmic localization of staining is evident; **f)** EBs at 19 days with non-immune control serum at 10x magnification.

Figure 3

Insulin secretion at varying glucose concentrations and growth conditions. a) Undifferentiated human embryonic stem cells (uhES) were cultured in knockout medium (n=6), or were allowed to differentiate in high-density adherent conditions (dhES) for 22 (n=12) and 31 days (n=7); **b)** hES grown in suspension as embryoid bodies (60-70 EBs/dish) for 20-22 days (n=6). Cultures were exposed to 3 ml serum-free medium for two hours, containing either 25 or 5.5 mM glucose as indicated. The media were harvested following incubation and insulin concentration measured

using enzyme immunoassay as described in methods. Data are represented as mean ± s.e.m. *, P<0.0001 *vs.* uhES. #, P=0.0004 *vs.* dhES-d22

Figure 4

Expression of β-cell related genes. Total RNA was extracted from undifferentiated human embryonic stem cells (uhES), differentiated hES growing either as embryoid bodies (EB) or as high-density adherent cell cultures (dhES) at various stages of differentiation, and from normal human fibroblasts (NHF). cDNA was synthesized from 7 μg total RNA and oligo dT primer. Aliquots of cDNA were diluted 1:2 for insulin and islet-specific glucokinase (GK), or 1:5 for Glut-1, Glut-2, Oct4, Ngn3 and IPF1/PDX1 prior to PCR. β-actin served as an internal standard. NC indicates no cDNA. For insulin, GK, Glut-2, Glut-1, Oct4, Ngn3, IPF1/PDX1 and β-actin, 36, 38, 40, 31, 37, 35, 35 and 28 cycles were applied, respectively. **a**) insulin, GK and β-actin ; **b**) Glut-1, Glut-2 and β-actin ; **c**) Oct4, Ngn3, IPF1/PDX1 and β-actin.

Detailed description of the invention

It is known in the art that embryonic development of the pancreas is the result of several distinct but interacting mechanisms involving growth factors, epithelial-mesenchymal interactions (31) and extracellular matrix that eventually regulate the expression of diverse transcription factors (27, 32, 33). However, the initial signal in the cascade of events that eventuate in the commitment of gut endoderm to develop into pancreatic tissue is still unknown.

According to the present invention we now provide evidence that a pathway for producing insulin-secreting cells with additional β-cell features is not an infrequent outcome in the course of spontaneous differentiation of human embryonic stem cells in culture, under the appropriate conditions as disclosed herein. This observation is a prerequisite for experimental strategies based upon the enrichment of spontaneously appearing β-cells or their precursors for cell replacement therapy. The cells described herein, produce and secrete insulin, and express two essential genes, Glut-2 and islet-specific glucokinase, that are believed to play an important role in β-cell function and glucose stimulated insulin secretion (34-36). The possibility that the insulin staining cells are unrelated to β-cells and are of extraembryonic or other origin (37) is highly unlikely, in view of the other markers identified, including the temporal course of appearance of beta cell developmental markers and in view of the secretion of fully processed insulin.

It is a second prerequisite for effective cell replacement therapy that the insulin production by human embryonic stem cells be glucose responsive or otherwise regulatable.

As long as β-cells are not in a homogeneous or enriched state, but rather present among other cell types, we cannot isolate the effects of glucose from countervening effects of other secretagogues. Furthermore, in the absence of homogeneous cell populations, comparisons based upon different experimental conditions are not readily quantified because of heterogeneity among EBs and the difficulty in normalizing insulin response to parameters such as protein or DNA content (38). The fine-tuning of insulin secretion in response to glucose requires cross talk between adjacent β-cells due to functional heterogeneity, and

it has been shown that isolated β-cells function differently compared to β-cells found in clusters or pseudoislets (39,40). Conceivably, long term exposure to high glucose levels which might affect the function of such insulin-producing cells and reduce their responsiveness to acute glucose changes as has been previously reported in other systems (38). This high glucose medium is needed to maintain the viable growth of hES in culture, but this does not preclude the possibility that protocols allowing growth of cells with insulin-producing capability in media containing lower glucose concentrations, may impart or restore glucose responsiveness. In any case, for treatment of diabetes with β-cell grafts derived from differentiated hES, it is necessary to demonstrate stimulus–secretion coupling after obtaining enriched or homogeneous β-cell cultures, as has been demonstrated for mouse ES derived β-cells (14).

Recently, islet cells were successfully generated *in vitro* from pancreatic stem cells (41,42) of humans and adult mice. In the latter study, Ramiya et al. have shown that these islet cells could reverse diabetes after being implanted in non-obese diabetic mice. However, the major practical limitation of this approach is the restricted number of cells that can be cultivated from human pancreata. Hence, human embryonic stem cells represent a reasonable potential alternative.

Currently used hES cells are not of clonal origin, despite their homogenous appearance in the undifferentiated state, suggesting the need to examine the *in vitro* differentiation of each hES derived cell line independently, or to examine clonal hES cell lines with well defined differentiated responses to growth factors (43).

In terms of strategies for enriching the population of insulin-secreting hES derived cell lines for further characterization and study, our results certainly indicate that the approach first described by Klug et al. to enrich cardiomyocytes from mouse ES cells is potentially applicable (44). As one non-limitative example, in the case of β-cells, the insulin promoter fused to a downstream selection marker could serve as the relevant selection tool. Indeed, recently this strategy was extended to enrichment of beta cells from mouse ES cells (14). Our findings indicate that even under conditions of spontaneous differentiation from non-clonal pluripotent hES cells, EBs are produced with a surprisingly high representation of cells with insulin producing capacity. In the current study more than 60% of EBs contained scattered pockets of positively staining cells that represented some 1-3% of the population of cells within the human EBs, as opposed to less than 1% in the mouse ES (6,14). Although this appears to occur spontaneously, it should be noted that the differentiation medium was supplemented with bFGF. Recently, Hart et al. (45) have suggested that FGF signaling may be involved in β-cell maturation, terminal differentiation and post-natal expansion. Tissue engineering estimates indicate that this is already a sufficient basis for enrichment protocols, based on the strategy described in Klug et al (44). Of importance, the finding that a subset of about 60% of the EBs contain insulin while the remainder do not, suggests that it may be useful to begin by selecting the subset of EBs with the highest percentage of insulin expressing cells as the source material for subsequent enrichment. Selecting this subset of EBs without sacrificing them, can be achieved using hES stably overexpressing a vital reporter marker driven by the insulin promoter. As exemplified herein below, additional protocols to enhance

the starting number of β-cells in the mixed population of cells within the EBs can improve the yield from enrichment strategies. Other limiting issues which may arise include possible senescence (46) of post differentiation hES cell derivatives, attributed to loss of telomerase, as we have reported (47).

As disclosed herein, the complex differentiation pattern of hES cells includes a subset of cells which have many characteristics of β-cell function, including proinsulin and/or insulin production and insulin release, as well as the expression of other β-cell markers. This finding is a necessary prerequisite for strategies based on cell enrichment from hES cells as a source of cell replacement in type 1 diabetes mellitus. Furthermore, the number of EBs with a surprisingly high percentage of such cells, is encouraging in terms of the potential prospects for successful use of insulin-producing human embryonic stem cells for cell replacement therapy.

Using RT-PCR applied to RNA extracted from differentiated hES cells, detection of a variety of differentiated cell markers including insulin has been reported (16). However, quantitative aspects, including elaboration of insulin into the medium, and percentage of insulin producing cells, were not known in the art. Such information is crucial to enable the use of hES cells as a source for β-cell replacement cell therapy.

For the first time these data are provided in the current disclosure, using the differentiation of the H9 line of hES cells. Using a variety of experimental approaches, we found abundant cells with beta cell features, including most notably insulin production and secretion.

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein. Rather the scope of the invention is defined by the claims which follow.

EXAMPLES

Research design and methods

Tissue culture. Large stocks of primary mouse embryonic fibroblasts (MEFs) were prepared as described by Robertson (17), and stored in liquid nitrogen. After each thaw, cells were used for only 3-5 passages.

The hES H9 cells were maintained in the undifferentiated state by propagation in culture on a feeder layer of MEFs that have been mitotically inactivated by γ-irradiation with 35 Gy and plated on gelatin coated six-well plates. Cells were grown in knockout DMEM (GIBCO/BRL, Grand Island, NY) supplemented with 20% serum replacement (GIBCO/BRL), 1% nonessential amino acids (GIBCO/BRL), 0.1 mM 2-mercaptoethanol (GIBCO/BRL), 1 mM glutamine (Biological Industries, Ashrat, Israel), 4 ng/ml human recombinant basic fibroblast growth factor (hrbFGF, PeproTech Inc, Rocky Hill, NJ). Cultures were grown in 5% CO2, 95% humidity and were routinely passaged every 4-5 days after disaggregation with 0.1% collagenase IV (Gibco/BRL).

Induction of differentiation in hES cells. Methods for the induction of differentiation in mouse ES were applied herein for the induction of hES differentiation (17,18). In brief, about 10^7 undifferentiated hES cells were

disaggregated and cultured in suspension in 100mm bacterial grade petri dishes (Greiner, Frickenhausen, Germany), which results in induction of synchronous differentiation, characterized by initial formation of small aggregates, followed by the acquisition of the configuration of embryoid bodies (EBs) (19). Alternatively, hES colonies were left unpassaged until confluence (about ten days), and then were replated on gelatinized six-well tissue culture plates in the absence of a feeder layer. The cells spontaneously differentiated to an array of cell phenotypes. The growth media that were used in differentiation were as described above.

Histological Analysis. EBs were collected at indicated intervals, washed three times with ice cold PBS, fixed overnight in 10% neutral buffered formalin, dehydrated in graduated alcohol (70-100%) and embedded in paraffin. For general histomorphology 5μm sections were stained with hematoxylin/eosin.

Immunohistochemistry. Deparaffinized 5 μm sections were incubated for 90 minutes at room temperature with the primary antibody: polyclonal guinea pig anti-swine insulin, 1:100 dilution (Dako), followed by incubation with goat anti rabbit biotinylated secondary antibody. Detection was accomplished using streptavidin-peroxidase conjugate and AEC (or DAB) as a substrate (Histostain-SP kit, Zymed Lab Inc., CA). Counterstaining was carried out with hematoxylin. Non-immune serum was used as a negative control, and normal human pancreas paraffin sections were used as positive controls.

Morphometric studies. To estimate the relative percentage of cells staining positively by immunohisochemistry, morphometric measurements were conducted as previously described (20).

Insulin detection assay. For adherent cells: MEFs, undifferentiated hES cells, cells which had differentiated spontaneously *in vitro* for more than 20 days were grown in 6 well plates. Cells were washed three times with serum free medium containing 25mM glucose, and incubated in 3 ml serum free medium for two hours. For suspended EBs, experiments were performed in 50mm bacterial grade petri dishes. Sixty to seventy EBs per plate were exposed to 3 ml serum free medium containing either 5.5 or 25 mM glucose. Subsequently conditioned media were collected and insulin levels were measured using a Microparticle Enzyme Immunoassay MEIA (Abbott AXSYM® system Insulin kit code B2D010) which detects human insulin, with no crossreactivaty to proinsulin or C-peptide.

RT- PCR. Total RNA was isolated from undifferentiated hES cells, and from *in vitro* differentiated hES cells growing either as EBs or as high-density cultures at various stages of differentiation.

cDNA was synthesized from 7 μg total RNA using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Promega) in 1x transcription buffer containing 0.5 μmol/l oligo $dT_{(12-18)}$ (GIBCO/BRL) and 400 μmol/l dNTPs. Aliquots of cDNA were diluted 1:5 for IPF1/PDX1, neurogenin 3 (Ngn3), octamer-binding transcription factor (Oct4), Glut-1 and Glut-2, or 1:2 for insulin and islet specific glucokinase (GK). Subsequent PCR reactions were carried as

follows: 2.5 µl (for IPF1/PDX1, Ngn3, Oct4) or 5 µl cDNA (for others), 1x PCR buffer, 400 µmol/l dNTPs, 100 ng of each primer pair and 1 U Taq polymerase. After initial hot start for 5 min, amplification continued with 28 cycles for β-actin, 31 cycles for Glut-1, 40 cycles for Glut-2, 38 cycles for glucokinase, 36 cycles for insulin, 37 cycles for Oct4, 35 cycles for IPF1/PDX1 and Ngn3. Denaturation steps were at 94^0C for 1 min, and annealing at 58, 52, 50, 67,62, 55, 52 and 60^0C, respectively, for 1 min, and extension at 72^0C for 1 min, and a final polymerization for 10 min. The amplified products were separated on 1.5% agarose gels. Each PCR reaction was performed in duplicate and under linear conditions. The forward and reverse primer sequences used for determination of human insulin, IPF1/PDX1, Ngn3 and β-actin were as follows: hIns: 5'-GCC TTT GTG AAC CAA CAC CTG-3', 5' GTT GCA GTA GTT CTC CAG CTG-3' (261 bp fragment); IPF1: 5'-CCC ATG GAT GAA GTC TAC C-3', 5'-GTC CTC CTC CTT TTT CCA C (262 bp fragment); Ngn3: 5' CTC GAG GGT AGA AAG GAT GAC GCC TC-3', 5'-ACG CGT GAA TGG GAT TAT GGG GTG GTG-3' (948 bp fragment); β-Actin: 5'-CAT CGT GGG CCG CTC TAG GCA C-3', 5'-CCG GCC AGC CAA GTC CAG GAC GG-3' (508 bp fragment), respectively. The primer sequences used for determination of Glut-1, Glut-2, GK and Oct4 were as previously described (21-23), the amplified fragments being 310, 398, 380 and 320 bps, respectively.

Statistics. Results are expressed as mean ± s.e.m, and comparisons conducted using the unpaired Student's *t*-test.

Results with the H9 line of human ES cells

According to one currently preferred embodiment, the H9 line of hES cells were used. These cells grow as homogeneous and undifferentiated colonies when they are propagated on a feeder layer of MEFs. Accordingly, spontaneous *in vitro* differentiation of H9 cells was investigated following removal of cells from the MEF feeder layer, using two different model systems. Cells grown under adherent conditions in tissue culture plates, in the absence of MEFs displayed a pleiotropic pattern with numerous morphologies, including neuronal-like, muscle-like, or glandular-like structures (data not shown). In contrast, *in vitro* differentiation in suspension culture, resulted in a more consistent pattern with the formation of discrete EBs. One day after transfer to bacterial-grade petri dishes, cells failed to adhere and formed small aggregates. Following three days under these conditions, EBs acquired a simple structure with primitive endodermal layers surrounding inner cells (Figure 1a), and then continued to grow in size and developed a more cystic structure (400-700μm). These are similar to the morphologies reported for mouse EBs (24). Subsequent studies were carried out using immunohistochemistry (IHC) to determine the spatial and temporal pattern and to obtain an estimate of the relative density of cells in suspension cultured EBs with insulin producing capability. Hematoxylin and eosin staining of paraffin embedded sections was used to provide the overall histological morphology of EBs. Organization of EBs started as early as day three following removal from MEFs and transfer to suspension culture. With progressive days in suspension culture, more complex structures became evident, such as epithelial or endothelial-like cells lining hollow structures or cysts (figure 1b-d).

Following EB development, they were collected every three days until day nineteen. Immunohistochemistry, using anti-insulin antibody, revealed cells expressing insulin as early as fourteen days of differentiation, with progressive increase in number through to day 19. Insulin expressing cells were found either scattered throughout the EBs or organized into small clusters (figure 1f-h). Among EBs which stained positively for insulin (60-70%), on average 1-3% of cells were positively staining at maximum density. The remaining 30-40% of EBs were negative for insulin staining.

In order to characterize these insulin-containing cells, which are interspersed among the mixed population of spontaneously differentiating adherent hES, insulin elaborated into the medium was measured by enzyme immunoassay in undifferentiated hES, differentiated hES, and MEF cells. Growth medium contained serum replacement and 25mM glucose, which is essential for hES viability. Insulin secretion was measured under both culture conditions of adherent cells, and from EBs. In adherent cells, insignificant immunoreactive insulin could be detected in serum-free media harvested from undifferentiated hES (5.6±0.6 μU/ml, n=6), and none from a feeder layer without overlying hES. However, in serum-free media harvested after 22 and 31 days of differentiation, insulin concentrations were as follows: 126.2±17.7 (n=12) and 315.9±47 μU/ml (n=7), respectively (figure 2a). Similarly, as shown in Figure 2b, insulin release was significantly higher from 20-22 day EBs (60-70 EBs per each experiment) as compared to uhES. In order to assess EB insulin responsiveness to different glucose concentrations, EBs containing culture dishes were acutely exposed to either 5.5 mM or 25mM of glucose for two hours. These acute changes in medium glucose did not elicit any significant

differences in elaborated insulin concentrations between the two groups (158±16 (n=6) *vs.* 146.2±22.1μU/ml (n=6), respectively, figure 2b).

The foregoing results prompted us to examine the expression of other β-cell related genes using RT-PCR analysis of total RNA extracted from undifferentiated and differentiated hES cells. As shown in figure 3, insulin mRNA was detected in differentiated cells but not undifferentiated hES. In parallel, islet glucokinase and Glut-2 genes were also identified following but not prior to differentiation. Similar results were obtained using either EBs or high-density adherent culture conditions. On the other hand, the Glut-1 isotype, a constitutive glucose transporter, was widely expressed in all forms of hES, as well as in human fibroblasts. Expression of three transcription factors was examined. As expected, mRNA expression of Oct4, a marker of the pluripotent state (25,26), was detected in undifferentiated hES, but decreased progressively during the subsequent three weeks of differentiation (figure 3c). We also demonstrated that differentiating hES cells express IPF1/PDX1 and Ngn3 transcription factors (figure 3c), that together have been shown to regulate pancreatic and endocrine cell differentiation (27-30).

Insulin producing cells from hTERT over-expressing hES cells.

Since pluripotent hES cells are capable of differentiating into many cell types, they or their derivatives can be used for research and medical applications, including cellular transplantation. A major objective of this invention is to modulate the differentiation of hES cells so that a desired population of precursors or fully differentiated cells can be obtained.

We have already provided evidence that a surprisingly high number of insulin producing β-cells appear during the differentiation of hES in culture, under the conditions that we have utilized. This observation is a prerequisite for experimental strategies based upon the enrichment of spontaneously appearing β-cells or precursors for tissue engineering and cell replacement therapy in the treatment of diabetes mellitus.

However, it is conceivable that enrichment and propagation of large number of such differentiated cells will suffer the disadvantage of low proliferation capacity and limited life span. To overcome this limitation, agents which may confer growth advantage (e.g. telomerase) may be utilized. Telomerase in particular may be used to maintain telomere length and integrity and thereby extend the proliferation capacity of the cells. In those instances where ectopic over-expression of hTERT does not adversely influence the differentiation pattern, it is possible to generate a fully differentiated desired lineage of telomerase positive cells.

Experimental protocol:

1). **Ectopic expression of hTERT in undifferentiated hES cells.** hTERT over-expressing hES cells are allowed to differentiate under the same conditions chosen for selection and enrichment for production of insulin producing cells (combination of factors such as insulin+transferrin+sodium selenite (ITS), glucose, nicotinamide, KGF, FGF, VEGF, EGF, NGF, activin, β-cellulin, in the culture medium). According to one currently most preferred embodiment of the invention it is now possible to significantly enhance the yield of insulin producing cells using the combination of factors known as ITS, namely insulin, transferrin and selenite. One

preferred salt is sodium selenite but other salts containing selenium may also be utilized.

We use the strategy of genetic modification of human ES cells using a cell specific promoter-neo^r transgene in a way that permits the generation of homogeneous insulin producing cells, by utilizing an appropriate (e.g. bicistronic) expression vector that contains an attenuated internal ribosome entry site (IRES). This vector is constructed so that hTERT coding sequence is driven by PDX-1, a cell specific gene promoter that becomes active in β-cells progenitors at the very early stages of β-cells differentiation pathway. Alternative promoters may substitute instead of that for PDX-1. The hTERT coding region is constructed to reside in a single cassette with an IRES and hygromycin or other antibiotic resistance selection marker. In addition this vector carries a neomycin (or other antibiotic) selection marker under the control of a constitutive promoter, including for example PGK promoter or another mammalian promoter. The construct is transfected into undifferentiated hES cells and neomycin resistant clones are selected. In the subsequent step, these clones are allowed to differentiate and hygromycin resistant clones are selected. Virtually, all the transfected cells, which express the selection marker, are expected to express telomerase and most likely differentiate into the insulin producing cell lineage that has the ability to activate the PDX-1 cell specific promoter at the very early stages of this differentiation pathway.

In order to prevent the toxic effect of high glucose on the PDX-1 promoter due to the high glucose concentration in the growing medium of hES cells, antioxidant reagents, including but not limited to Nicotinamide, L-ribose, N-acetyl-cysteine, and others antioxidants are added to the culture medium.

2) Criteria for Selection of clones

The resultant clones are examined for advantageous properties and/or lack of deleterious properties, as follows:

1. Examination at the undifferentiated state for expression of the Neo^r gene using RT-PCR to avoid proceeding with the selection procedure with false positive clones.

2. Examination of the resultant clones following differentiation and hygromycin selection for expression of exogenous hTERT or other proliferation (extended life-span) promoting gene using northern blot analysis or RT-PCR, for Telomerase activity using TRAP assay and for telomere length using TRF assay. The above are monitored at multiple time intervals following differentiation. As control, normal hES that went through the same process are used.

3. Examination of the expression profile of factors involved in β-cells differentiation and maturation using quantitative RT-PCR. The expression of the following genes are monitored: Insulin, PDX-1, Nkx6.1, Nkx2.2, Glut-2, Pax-6, BETA2, Ngn3, Islet-1, Pax-4, Hlxb-9, GK, Nestin, Prohormone convertase (PC) 1 and 2, glucagons, GAD65. The pattern of expression is compared with control cells. A microchip for expression profiling is used to facilitate the screen, if needed.

4. Examination of specific enzyme profile using enzymatic assays for Glut2, GK, PC.

5. Quality control of insulin producing cells derived as above from hES cells will be carried out using the following parameters: ultrastructural characterization using electron microscope, insulin secretion, insulin processing, electrophysiological profile, metabolic profile.

6. Compare β-cells developmental gene expression profile in the clones versus normal cells using microarray chips.

7. Immunohistochemistry or western blot analysis using specific antibodies for β-cells specific markers.

8. Lack of tumorigenic properties are examined according to any of the criteria as are known in the art, including the ability to generate tumors in nude mice, the ability to grow on soft agar (focus formation), and presence of a normal cell karyotype.

3). <u>Stable transfection of hTERT into fully differentiated cell lineage.</u>

Following enrichment and characterization of a fully differentiated cell lineage, e.g. insulin producing β-cells, hTERT gene coding sequence driven by a powerful promoter such as the β-actin gene promoter or PGK gene promoter, are stably transfected with a selection marker, to overcome the disadvantage of low proliferation capacity and limited life span. This strategy is to promote proliferation of insulin producing β-cells that might replace the necessity of donors for islet transplantation. The final clonal population is examined for all the criteria mentioned above. In parallel, this clonal population is examined for telomerase activity, telomere length, extension of life span and lack of tumorigenic properties as outlined above.

4) <u>Ectopic expression of hTERT and /or growth enhancing gene in undifferentiated hES cells in a combination with Cre-recombinase gene.</u>

For establishing a large population of insulin producing cells for cell therapy, eventually it is necessary to overcome the problems of limited life span and

proliferation capacity of mature finally differentiated β-cells. As described above, to overcome this limitation telomerase activity may be utilized to maintain telomere length and integrity and thereby extend the proliferation capacity of the cells. Alternatively, a growth-enhancing gene can be inserted into the hES cells similar to the strategy described above. In brief, the coding region of a growth enhancing gene such as SV40 large T-antigen, under the control of PDX-1 promoter is constructed in a single cassette with an IRES and hygromycin selection marker in a bicistronic vector that carries also neomycin resistant gene under the control of a constitutive promoter of the β-actin gene or the PGK gene. Progenitor cell populations are allowed to expand for several orders of magnitude following induction of terminal differentiation into mature β-cells. These mature cells are examined using the criteria described above with an emphasis on criterion number 8, namely the lack of tumorigenicity.

For the purpose of cell transplantation DNA sequences with the potential to develop tumorigenicity are removed from the cells prior to transplantation. hTERT gene is not an oncogene, but its reactivation occurs in 85-95% of the cancers. In addition most of the growth enhancing genes have the capacity to confer malignant characteristic of the cells.

For this purpose we utilize the Cre recombinase-loxP (Cre-loxP) system to confine the inactivation and elimination of the target sequences in a cell specific manner. Cre-recombinase functions as a site specific recombinase, splicing DNA sequences between specific 34-bp sequences known as loxP sites. The unique property of the insulin gene to be expressed only in pancreatic β-cells, allows using the β-cells specific insulin promoter for Cre recombinase expression to inactivate and eliminate genes in fully matured β-cells. The protocol uses stably transfect hES

with hTERT or a growth enhancing gene coding sequences under the control of β-cells progenitor cell specific promoter PDX-1 as described above in a single cassette with an IRES and hygromycin or other antibiotic selection marker. *lac*Z gene coding sequence inserted independently downstream of the hygromycin gene will remain unexpressed in the progenitor cells as long as Cre recombinase is not activated. The hTERT-IRES-Hygromycin sequences are flanked by loxP (floxed) to allow excision of this sequence following expression of Cre recombinase in the cells. Upon activation of insulin promoter and thereby the Cre recombinase gene in fully matured β-cells, this sequences is floxed out allowing the *lac*Z coding sequence to be expressed under the control of the PDX-1 promoter. The expression of the *lac*Z gene depends on the expression of the Cre recombinase, therefore a time course study is essential to determine the optimal time required for maximum recombination to excise the target gene in β-cells that results in expression of the *lac*Z gene.

Measurement of *lac*Z enzyme activity is carried out using a fluorogenic substrate, which is hydrolyzed and retained intracellularly. The advantage of this system is that *lac*Z serves both as a reporter gene to quantitate recombination efficiency and as a selectable marker for the fluorescence-activated sorting of cells based on their *lac*Z expression level.

Another variant of this approach, utilizes the Cre-recombinase driven by the insulin promoter in cells in which the thymidine kinase gene has been stably transfected in between loxP sites. Cell toxicity is induced by addition of ganciclovir, and only those cells in which the TK is specifically floxed out (cell specific promoter driving Cre-recombinase) survive the ganciclovir treatment.

It will be appreciated by the artisan in the field to which this invention pertains that all of the foregoing protocols may be generalized to other cell-specific promoter based systems.

5) Glucose responsiveness

For glucose responsiveness our protocols utilize pre-incubations in media with varying glucose concentrations, with or without the addition of antioxidants. As a measure of appropriate incubation glucose concentrations for longer-terms culture, PDX-1 binding by EMSA is monitored.

Of importance, the finding that a subset of about 60% of the EBs contain insulin while the remainder do not, suggests that it may be useful to begin by selecting the subset of EBs with the highest percentage of insulin expressing cells as the source material for subsequent enrichment. Selecting this subset of EBs without sacrificing them, can be achieved using hES stably overexpressing a vital reporter marker driven by the insulin promoter.

Alternatively, the EBs population will be seeded in 96 well plates; one EB per well and insulin responsiveness will be demonstrated by measuring insulin secretion to the medium.

The specific growth conditions described above, or components thereof, are those which constitute the basis of protocols for promoting the favorable growth of insulin producing cells from hES. It will clearly be recognized by the skilled artisan that it is possible to change many of the particulars of these protocols without

departing from the essentials of the invention. All of the many possible variants may be considered optimization of parameters within the framework of the invention disclosed. The scope of the invention is defined by the claims which follow.

5

References:

1. The Diabetes Control and Complication Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Eng J Med* 329:977-986 (1993)

2. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M: Reversal of lesions of diabetic nephropathy after pancreas transplantation. *N Eng J Med* 339:69-75 (1998)

3. Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Eng J Med* 343:230-238 (2000)

4. Samstein B, Platt JL: Physiologic and immunologic hurdles to xenotransplantation. *J Am Soc Nephrol* 12:182-193 (2001)

5. Efrat, S. Prospects for gene therapy of insulin-dependent diabetes mellitus. *Diabetologia* 41:1401-9 (1998)

6. Efrat S: Genetically engineered pancreatic β-cell lines for cell therapy of diabetes. *Ann N Y Acad Sci* 875:286-93 (1999)

7. Soria B, Andreu E, Berná G, Fuentes E, Gil A, León-Quinto T, Martín F, Montaya E, Nadal A, Reig JA, Ripoll C, Roche E, Sanches-Andrés J: Engineering Pancreatic islets. *Pflügers Arch-Eur J Physiol* 440:1-18 (2000)

8. Cheung AT, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, Kieffer TJ: Glucose-dependent insulin release from genetically engineered K cells. *Science* 290:1959-1962 (2000)

9. Lee HC, Kim SJ, Kim KS, Shin HC, Yoon JW: Remission in models of type 1 diabetes by gene therapy using a single chain insulin-analogue. *Nature* 408:483-488 (2000)

10. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A: Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat Med* 6:568-572 (2000)

11. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ Marshal VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-7(1998)

12. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A: Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotech* 18:399-404 (2000)

13. Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD: Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95: 13726-13731 (1998)

14. Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martín F: Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 49:157-161 (2000)

15. Shamblott MJ, Axelman J, Littlefield JW, Blumenthal PD, Huggins GR, Cui Y, Cheng L, Gearhart JD: Human embryonic germ cell derivatives express a broad

range of developmentally distinct markers and proliferate extensively *in vitro*. *Proc Natl Acad Sci USA* 98: 113-118 (2001)

16.Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 97:11307-12 (2000)

17.Robertson E: Teratocarcinomas and embryonic stem cells a practical approach. Practical approach series. IRL press. 71-112 (1987)

18.Keller GM: *In vitro* differentiation of embryonic stem cells. *Curr Op Cell Biol* 7:862-9 (1995)

19.Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N: Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6:88-95 (2000).

20.Green J, Maor G: Effect of metabolic acidosis on the growth hormone/IGF endocrine axis in skeletal growth centers. *Kidney Int* 57:2258-2267, 2000

21.Seino, Y. *et al.* Abnormal facilitative glucose transporter gene expression in human islet cell tumors. *J Clin Endocrinol Metab* 76:75-8 (1993)

22.Koranyi LI, Tanizawa Y, Welling CM, Rabin DU, Permutt MA: Human islet glucokinase gene. Isolation and sequence analysis of full-length cDNA. *Diabetes* 41:807-11 (1992)

23.Van Eijk MJ, Van Rooijen MA, Modina S, Scesi L, Folkers G, Van Tol HT, Bevers MM, Fisher SR, Lewin HA, Rakacolli D, Galli C, de Vaureix C, Trounson AO, Mummery CL, Gandolfi F: Molecular cloning, genetic Mapping and developmental expression of bovine POU5F1. *Biol Reprod* 60:1093-1103 (1999)

24.Abe K: Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies. *Exp Cell Res* 229:27-34 (1996)

25.Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Kazuyuki O, Gross M, Hübner K, Schöler HR: Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* 122:881-894 (1996)

26.Niwa H, Miyazaki JI, Smith AG: Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat genet* 24:372-376 (2000)

27.Edlund, H: Transcribing pancreas. *Diabetes* 47:1817-23 (1998)

28.Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendhal U, Edlund H: Notch signalling controls pancreatic cell differentiation. *Nature* 400:877-881 (1999)

29.Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS: Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* 127:3533-3542 (2000)

30.Herrera PL: Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* 127:2317-2322 (2000)

31.Scharfmann R: Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. *Diabetologia* 43:1083-1092 (2000)

32.Edlund H: Pancreas: how to get there from the gut? *Curr Op Cell Biol* 11:663-8 (1999)

33.Kim SK, Hebrok M: Intercellular signals regulating pancreas development and function. *Genes & Dev* 15:111-127 (2001)

34.Efrat S: Making sense of glucose sensing. *Nat genet* 17:249-250 (1997)

35.Matschinsky FM, Glaser B, Magnuson MA: Pancreatic β-cell glucokinase. Closing the gap between theoretical concepts and experimental realities. *Diabetes* 47:307-315 (1998)

36.Shepherd PR, Kahn BB: Mechanism of disease: glucose transporters and insulin action. Implication for insulin resistance and diabetes mellitus. *N Eng J Med* 341:248-57 (1999)

37.Alarcón C, Serna J, Pérez-Villamil B, de Pablo F: Synthesis and differentially regulated processing of proinsulin in developing chick pancreas, liver and neuroretina. *FEBS Letters* 436:361-366 (1998)

38.Ling Z, Pipeleers DG: Prolonged exposure of human β cells to elevated glucose levels results in sustained cellular activation leading to a loss of glucose regulation. *J Clin Inv* 98:2805-12 (1996)

39.Jonkers FC, Jonas JC, Gilon P, Henquin JC: Influence of cell number on the characteristics and synchrony of Ca^{2+} oscillations in clusters of mouse pancreatic islet cells. *J Physiol* 520:839-49 (1999)

40.Charollais A, Gjinovci A, Huarte J, Bauquis J, Nadal A, Martín F, Andreu E, Sánches-Andrés J, Calabrese A, Bosco D, Soria B, Wolheim CB, Herrera PL, Meda P: Junctional communication of pancreatic β cells contributes to the control of insulin secretion and glucose tolerance. *J Clin Invest* 106:235-243 (2000)

41.Bonner-Weir S, Taneja M, Weir GC, Tatarkiewics K, Song KH, Sharma A, O'Neil JJ: *In vitro* cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci USA* 97:7999-8004 (2000).

42.Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG: Reversal of insulin-dependent diabetes using islet cells generated *in vitro* from pancreatic stem cells. *Nat Med* 6:278-282 (2000).

43.Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA: Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 227:271-278 (2000)

44.Klug MG, Soonpaa MH, Koh GY, Field LJ: Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Inv* 98: 216-24 (1996).

45.Hart AW, Baeza N, Apelqvist Å, Edlund H: Attenuation of FGF signaling in mouse β-cells leads to diabetes. *Nature* 408:864-868 (2000)

46.Halvorsen TL, Beattie GM, Lopez AD, Hayek A, Levine F: Accelerated telomere shortening and senescence in human pancreatic islet cells stimulated to divide *in vitro*. *J Endocrinol* 166:103-109 (2000)

47.Tzukerman M, Shachaf C, Ravel Y, Braunstein I, Cohen-Barak O, Yalon-Hacohen M, Skorecki KL: Identification of a novel transcription factor binding element involved in the regulation by differentiation of the human telomerase (hTERT) promoter. *Mol Biol Cell* 11:4381-4391(2000)

CLAIMS

1. A cell population comprising insulin-producing cells derived from human embryonic stem cells.
2. The cell population of claim 1 enriched for insulin-producing cells derived from human embryonic stem cells.
3. The cell population of claim 2 wherein the enrichment comprises treatment of the human embryonic stem cells with insulin, transferrin and selenite.
4. The cell population of claim 1 comprising selected insulin-producing cells derived from human embryonic stem cells.
5. The cell population of claim 1 comprising isolated insulin-producing cells derived from human embryonic stem cells.
6. The cell population of claim 1 comprising cloned insulin-producing cells derived from human embryonic stem cells.
7. A cell population comprising regulatable insulin-producing cells derived from human embryonic stem cells.
8. The cell population of claim 7 comprising glucose-responsive insulin-producing cells derived from human embryonic stem cells.
9. The cell population of claim 8 enriched for glucose-responsive insulin-producing cells derived from human embryonic stem cells.
10. The cell population of claim 9 wherein the enrichment comprises treatment with insulin, transferrin and selenite
11. The cell population of claim 8 comprising selected glucose-responsive insulin-producing cells derived from human embryonic stem cells.

12. The cell population of claim 8 comprising isolated glucose responsive insulin-producing cells derived from human embryonic stem cells.

13. The cell population of claim 8 comprising cloned glucose-responsive insulin-producing cells derived from human embryonic stem cells.

14. A cell population comprising stable insulin-producing cells derived from human embryonic stem cells.

15. The cell population of claim 12 comprising stable clonal insulin-producing cells derived from human embryonic stem cells.

16. The cell population of claim 12 comprising insulin-producing cells derived from human embryonic stem cells overexpressing hTERT.

17. Use of insulin producing cells derived from human embryonic stem cells for cell replacement therapy.

18. Use according to claim 15 wherein the cells are transplanted into a pancreas of a subject in need thereof.

19. Use according to claim 15 wherein the cells are transplanted to an ectopic site in a subject in need thereof.

20. A method of treating a patient in need thereof with insulin producing cells derived from human embryonic stem cells comprising transplantation of a cell population comprising insulin producing human embryonic stem cells.

21. A cell population comprising committed precursors of β islet cells of the pancreas derived from human embryonic stem cells.

for the applicant

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ABSTRACT

The present invention discloses insulin producing cells derived from human embryonic stem cells. Methods of generating, enriching, selecting and isolating populations of insulin-producing embryonic cell lines are disclosed. Insulin production in these cells can be glucose responsive, or otherwise regulatable. Advantageously, these insulin-producing cells can be stable cell lines. Methods of using these cells, cell populations or cell lines particularly for cell therapy are disclosed.

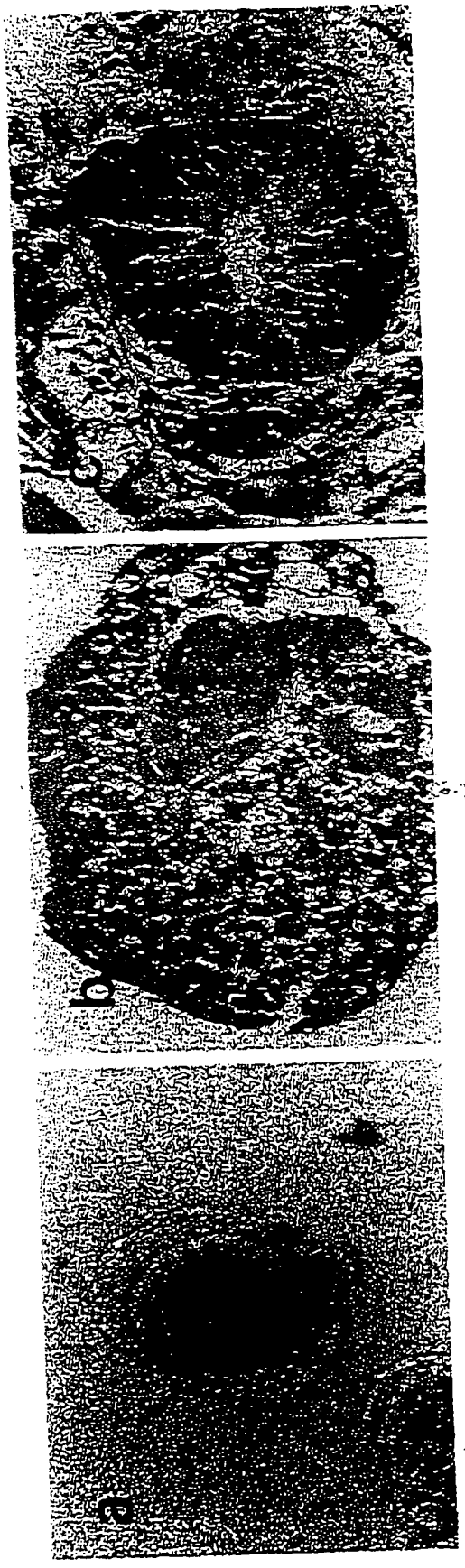


FIGURE 1

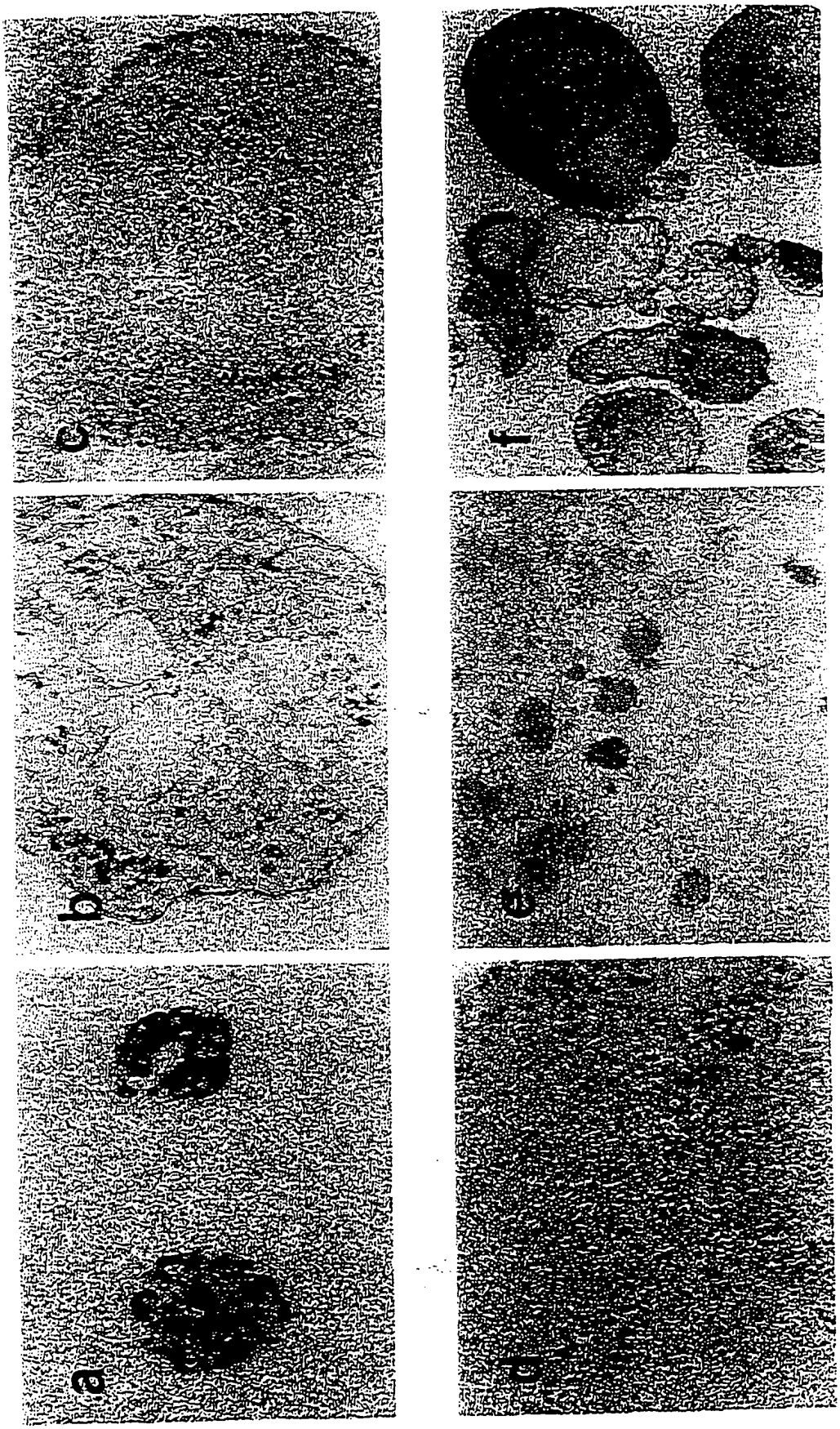


FIGURE 2

FIGURE 3

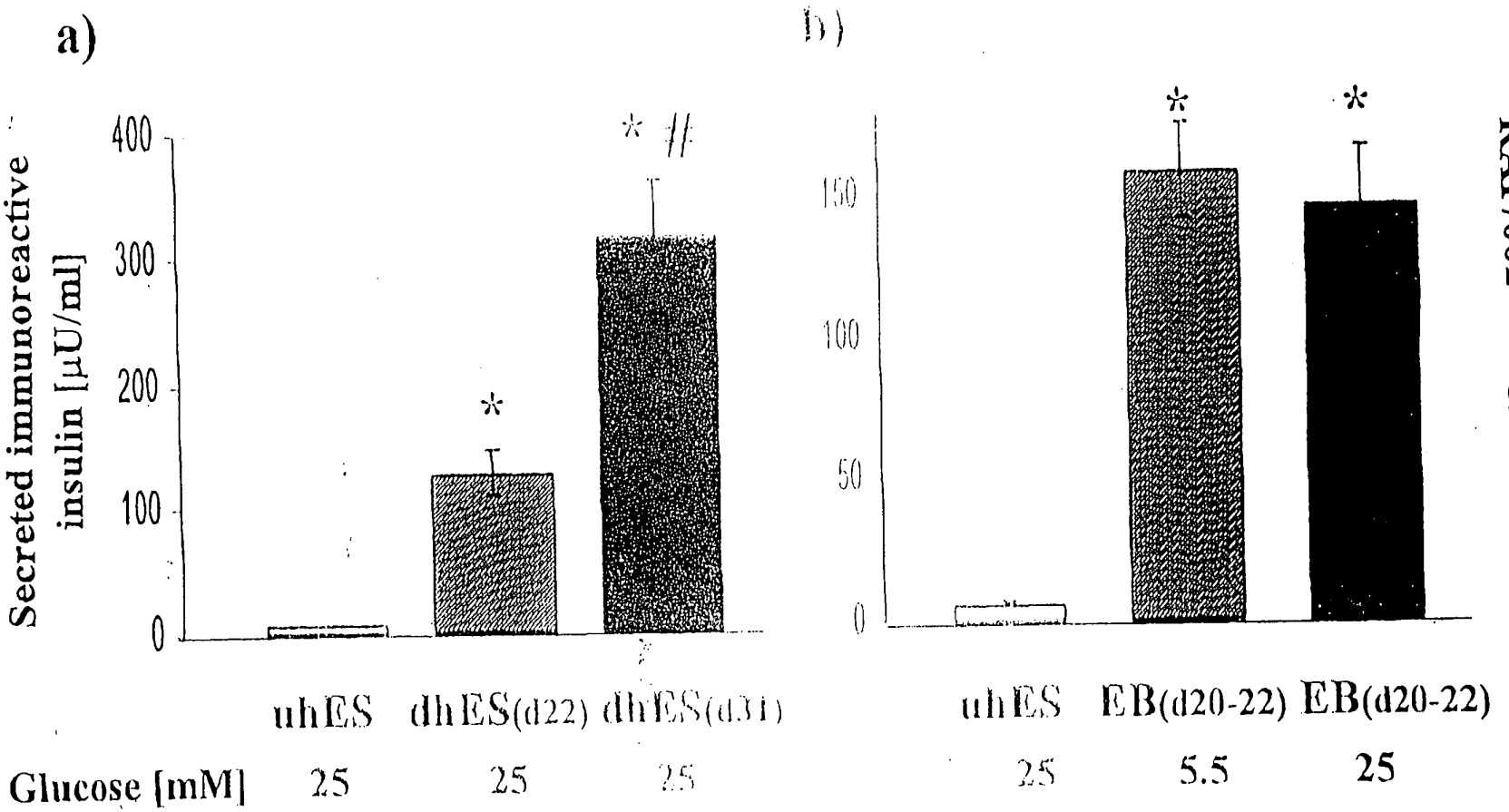
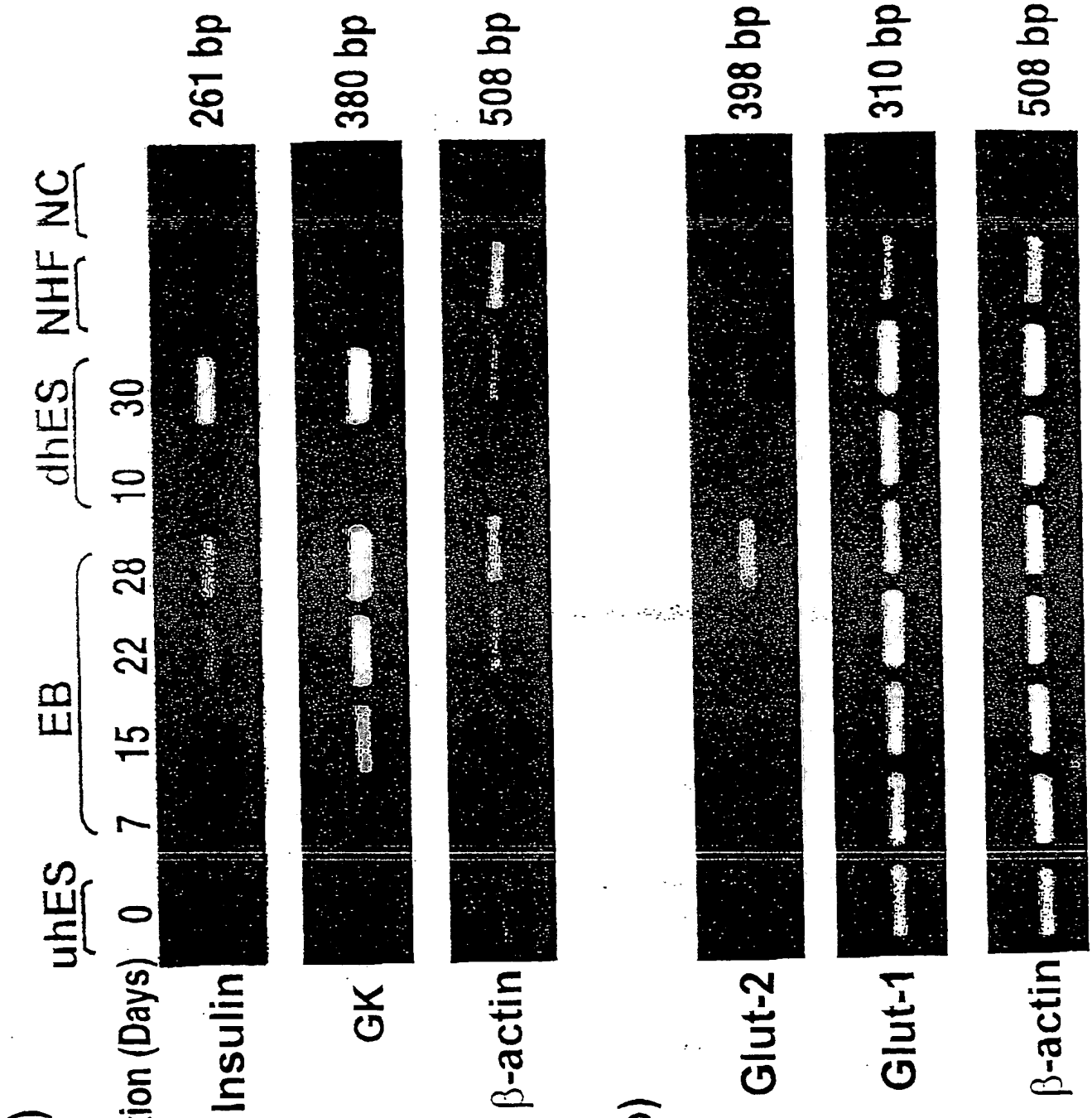

a)
b)
Secreted immunoreactive insulin [µU/ml]
400
300
200
100
0
150
100
50
0
*
*
*
//
uhES
dhES(d22)
dhES(d31)
uhES
EB(d20-22)
EB(d20-22)
Glucose [mM]
25
25
25
25
5.5
25

FIGURE 4


a)
uhES
EB
dhES
NHF
NC
Differentiation (Days)
0
7
15
22
28
10
30
Insulin
261 bp
GK
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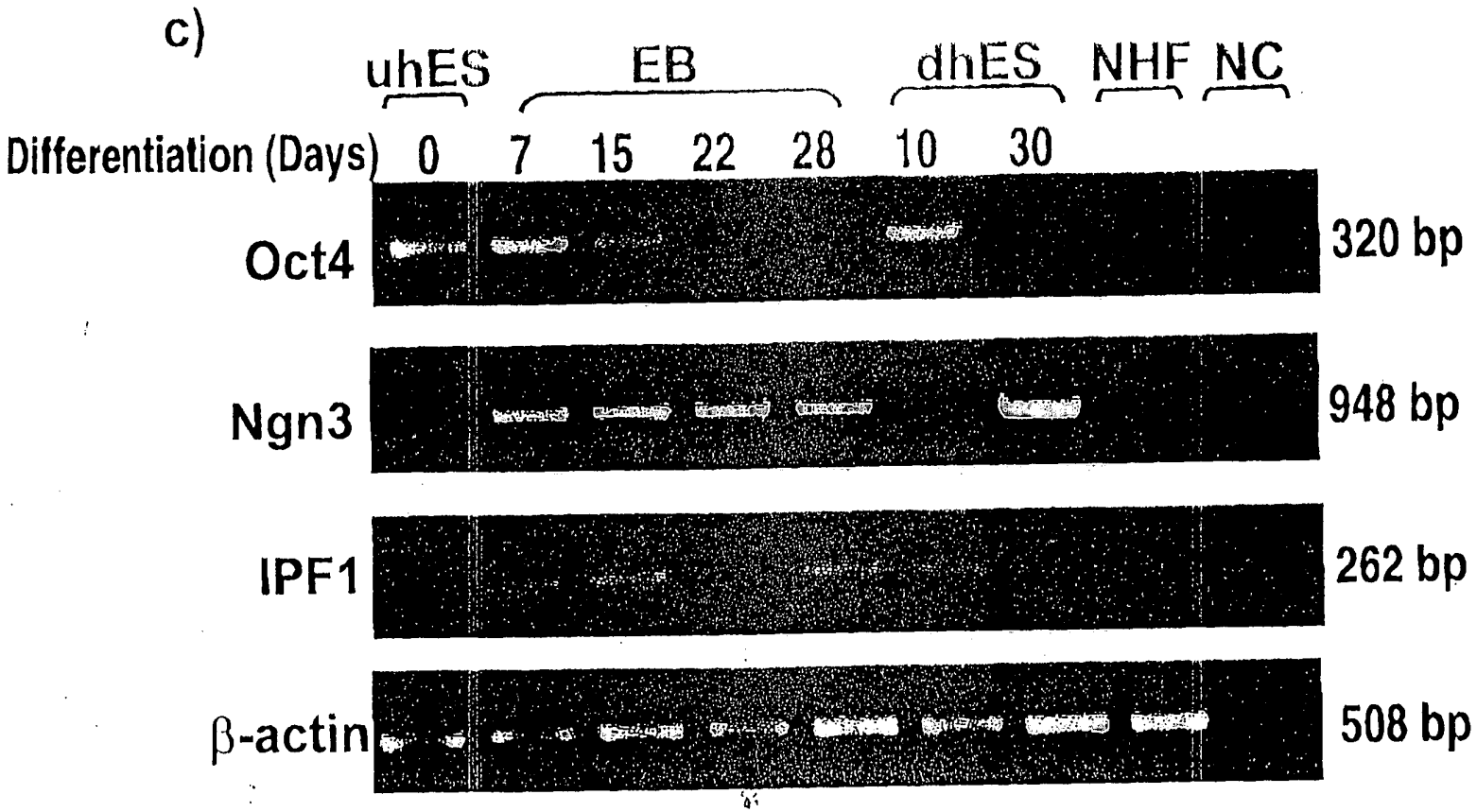
β-actin
508 bp
b)
Glut-2
398 bp
Glut-1
310 bp
β-actin
508 bp

FIGURE 5


c)
uhES
EB
dhES
NHF
NC
Differentiation (Days)
0
7
15
22
28
10
30
Oct4
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Ngn3
948 bp
IPF1
262 bp
β-actin
508 bp